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ANALYSIS OF DIFFERENT MANIFESTATIONS AND CAUSES OF LIQUID CULTURE OF *GANODERMA LUCIDUM* WITH THREE TRADITIONAL CHINESE MEDICINAL HERBS

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Abstract

Traditional Chinese medicine (TCM) has been in use since ancient times, most TCM is consumed directly without going through the extraction and purification process, several components are difficult to digest and absorb, such as cellulose, hemicellulose, and lignin. Some fungus (e.g. Ganoderma lucidum) can break down these difficult-to-use components in TCM, allowing the beneficial elements to be released more easily. As a result, these fungi had been studied and utilized in TCM research and development, Chinese herbal cosmetics development, and Chinese medicine waste utilization.After Ganoderma lucidum co-cultivation with various TCM, however, the degradation process and culture results may change. As a result, more research is required to understand the culture patterns of Ganoderma lucidum when cultivated with various herbal medicines. Ganoderma lucidum was cultivated with varied doses of ginseng root, reed rhizome, and kirilow rhodiola root in this experiment (3g, 5g, and 7g). The changes in fungal biomass and the amount of medication residue left in the medium were compared and studied after multiple days (3 days, 6 days, or 9 days) of incubation. The results showed that the *Ganoderma lucidum*- ginseng culture was superior to *Ganoderma lucidum*- phragmites, while the mycelial growth of *Ganoderma lucidum* was weaker in the rhodiola system. Electron micrographs showed that in the *Ganoderma lucidum*- ginseng culture, there were many mycelia attached to the surface of the ginseng powder, slightly less on the phragmites, and none could be seen on the rhodiola. The cellulose, hemicellulose, and lignin were most degraded in the ginseng system residue, followed by phragmites, and least in rhodiola. This may be the reason for the *Ganoderma* grow best in the ginseng system.

Key words: *Ganoderma lucidum*, Ginseng root, Reed rhizome, Root of kirilow rhodiola.

Introduction

Ganoderma lucidum is a 2,000 years old Chinese herb. The Pharmacopoeia of the People's Republic of China includes *Ganoderma lucidum* (Leyss. ex Fr.) Karst. and *Ganoderma sinense* of the family Polyporaceae (National Pharmacopoeia Committee 2015).

Both traditional and modern Chinese medicine recommend *Ganoderma lucidum* for its beneficial immunological effects, which led to its inclusion in the United States Pharmacopoeia (United States Pharmacopoeia 39-National Formulary 34 2016). As a fungus that is both edible and medicinal, *Ganoderma lucidum* is available in both fruiting body and mycelium forms and can be cultured in solid and liquid culture.

Therefore, it can perform incubation with other traditional Chinese medicines, forming new research directions such as secondary development of traditional Chinese medicines and new drug development (Zhang *et al*., 2021; Wang *et al*., 2020). The solid culture of Chinese medicines has been used for ages. For instance, more than a millennium ago, the ancient people in China used solid culture technology to prepare red yeast, Shen Qu (medicated leaven), pinellia, and agarwood (Zhang *et al*., 2021). In the 1990s, Zhuang *et al*., (2004) proposed the concept of "bi-directional fermentation", which led to the modernization of solid and liquid culture of traditional Chinese medicine (Zhuang *et al*. 2004; Zhuang 2010; Yi *et al*., 2007). This bi-directional culture refers to using Chinese herbs or residues with specific active ingredients as a medicinal substrate instead of the conventional nutritional substrate and the addition of selected strains of bacteria or fungi for biotransformation, constituting a culture combination called a fungous substance containing herbs or medicinal mycoplasm. Its bi-directional nature is reflected in the fact that the medicinal matrix, while providing the nutrients required by the fungi, is also influenced by the enzymes in the fungi to change their tissues and composition and produce new property and flavor functions (Zhang, 2005).

Many studies have been carried out on the liquid culture of Chinese herbal medicines using *Ganoderma lucidum* (Xin *et al*., 2018; Pei *et al*., 2019; Hu *et al*., 2020). Moreover, the mycelium, a liquid culture product of fungus, also possesses various pharmacological activities and has been used both for clinical treatment (Hsu & Cheng, 2018 In particular, the production conditions of mycelium in the fungus *Ganoderma lucidum* are easy to control and richer in polysaccharides. At the same time, cellulose, lignin, and other components are lower, facilitating human digestion and absorption. Therefore, it is prepared into medicines or food supplements.

Previous liquid culture of *Ganoderma lucidum* with TCM have mostly focused on changes in the post-culture product (mycoplasm) and promoted the release of active ingredients in the mycoplasm through liquid culture (Xin *et al*., 2018; Pei *et al*., 2019; Hu *et al*., 2020). However, it should be noted that the same fungus with different culture substrates will not give the same results, and the processes and products that result from the use of different herbs and *Ganoderma* may also be different. Relatively little research has been done on the culture conditions themselves, the interaction between the herbs and the *Ganoderma*: the consumption method of the medicine, the description of the growth process of the mycelium, and, in particular, there have been very few studies on its causes. For these reasons, three herbaceous and woody herbal medicines: ginseng root (Jia *et al*., 2017; Hsu *et al*., 2013), reed rhizome (Ning *et al*., 2017; Wu *et al*., 2019), and root of kirilow rhodiola (Wang *et al*., 2015; Cui *et al*., 2016), all of which have had undergone studies for liquid culture with fungi, were selected for comparative study. The objective of the study is to analyse the changes of medicinal mycoplasm and their causes in the culture systems of the three herbal medicines with *Ganoderma lucidum* under the same liquid culture conditions to provide a basis for the alternative herbal properties. This is required to investigate the role of *Ganoderma lucidum* in the liquid culture of herbal medicines, and to provide an insight into the performance and causes of fungi-herbal biotransformation.

Materials and Methods

Materials: The culture fungus of choice was *Ganoderma lucidum* (G. lucidum, cgmcc 5.0026), and the herbs of choice were ginseng root (*Panax ginseng* C. A. Mey. [P.schin-seng Nees], expressed as ginseng below), reed rhizome (*Phragmites communis* Trin. [*P. australis* (Cav.) Trin.], expressed as phragmites below), and root of *Kirilow rhodiola* (*Rhodiola sachalinensis* A. Bor. [*Sedum sachalinensis* (A. Bor.) Vorosh.], expressed as rhodiola below). Solarbio's cellulose, hemicellulose, and lignin assay kits were used for the detection of total sugar, glucose, xylose content, cellulose, hemicellulose, lignin, etc.

Methodology

Liquid culture process of *Ganoderma lucidum*-Chinese medicine: Prepared PDB liquid cultures and divided them into 250 ml conical flasks. Took 500g each of ginseng, rhodiola and phragmites and crushed in a Chinese herbal grinder, passed through a 120-mesh sieve, and then added to PDB culture at the dosages of 3g/bottle, 5g/bottle, and 7g/bottle. A total of 9 bottles per dose were made and repeated for three times. The bottles were then sealed and sterilized at 121°C for 30 minutes. The *Ganoderma* strains were incubated for 3 days, 6 days, and 9 days. The relevant experimental indicators were measured, each group was measured 3 times, and the average value was taken.

Determination of *Ganoderma lucidum* mycelial biomass: The *Ganoderma*-herb medium was centrifuged at 4800 r/min for 5min, and the supernatant was poured back into the corresponding conical flask. The precipitate was poured into a 40-mesh sieve, shook, and rinsed with water to wash away the medicine residue and residual medium. It was freeze-dried, and after drying, the dry weight of the corresponding *Ganoderma lucidum* mycelium was measured.

Scanning electron microscopy of pharmaceutical residues: After freeze-drying and spraying with gold, the surface morphology of the cultured herbal residue was analyzed using a Carl Zeiss EVO-18 scanning electron microscope.

Results

Comparison of *Ganoderma* mycelia of three *Ganoderma lucidum*- TCM liquid culture systems: The morphology and mycelial biomass of cultured *Ganoderma lucidum* were compared and analyzed between the experimental group with Chinese medicine and the control group without Chinese medicine. The sample groups were incubated with 3g/bottle, 5g/bottle, 7g/bottle of ginseng, rhodiola, and phragmites powders, respectively, for 3, 6, and 9 days (Figs. 1-2). Due to the small size of mycelium in the rhodiola medium, it is not easy to distinguish it from the residue, thereby the small amount of mycelium is not shown in the figure.

When compared with the control group, the mycelium pellets of liquid cultured *Ganoderma lucidum* mycelium with ginseng are firmer, smaller, and more viscous (Fig. 1).

Furthermore, the dry weight of *Ganoderma lucidum* mycelium with ginseng is heavier; that the mycelial biomass of liquid culture is larger, and that the biomass increases with time (Fig. 2). The addition of 5g/bottle of ginseng seemed to be more beneficial to the growth of *Ganoderma mycelium* mycelia.

Differences in mycoplasmic composition of the three culture systems: We further compared the changes over time of the medicinal mycoplasm in the three traditional Chinese medicine culture systems with different concentrations, including the quality of the residual medicine residue after incubation (Fig. 3), the content of starch, glucose, and total sugars present in the medium in the culture system, and the content of xylose, cellulose, hemicellulose, and lignin remaining in the medium in the Chinese medicine residue (Figs. 4 and 5).

Changes in the quality of the three herbal residues after incubation: The mass of residual residue of all three herbs decreased with time through the liquid culture of *Ganoderma lucidum*, but the level of change varied significantly (Fig. 3). Among them, the quality of ginseng residues was the least, followed by that of phragmites. The quality of rhodiola residues was the highest. It can be seen that during the liquid culture process, *Ganoderma lucidum* degradation was most visible with ginseng, followed by phragmites, with rhodiola showing the most negligible degradation.

Changes in the content of mycoplasmic carbohydrates and lignin in three *Ganoderma*-herbal culture systems: Generalized carbohydrates include soluble (eg. glucose and soluble starch) and insoluble saccharides (eg. insoluble starch and cellulose) which are an essential nutrient in the medium for *Ganoderma lucidum* mycelia, and the quantity contained is equally an essential factor in its growth and development. To determine whether the addition of different herbs would cause facilitation or inhibition of the utilization of sugars in the system, we compared the starch, glucose, and total sugar contents in the culture medium. We tested the cellulose, hemicellulose, and lignin contents in the remaining residue to determine whether different doses of the three herbs would affect the medicinal mycoplasm (including the culture medium and the components of the drug residue) during the culture process over time (Figs. 4 and 5).

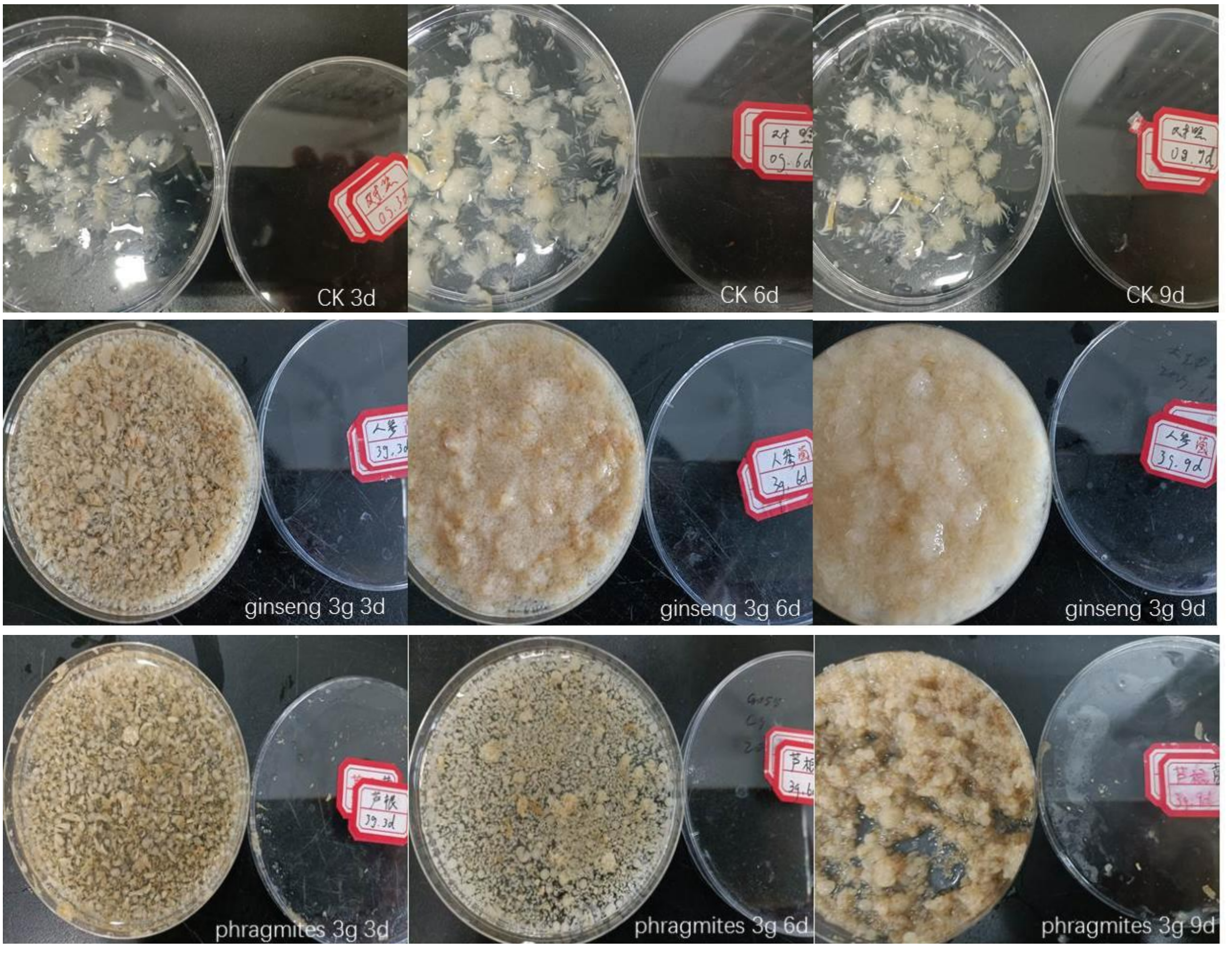


Fig. 1. Mycelial morphology of liquid cultured *Ganoderma lucidum* in different treatment groups.

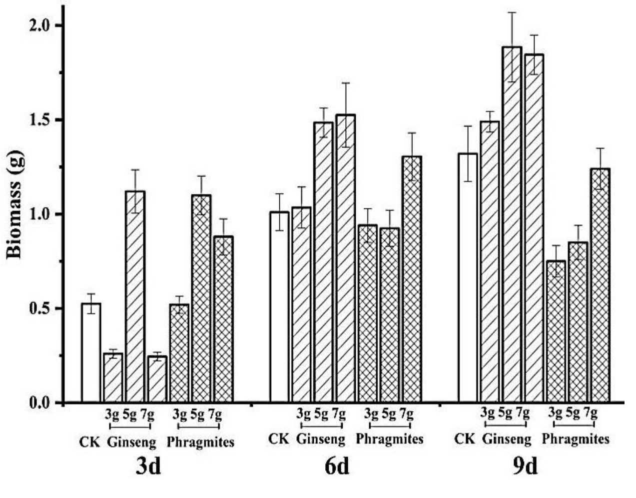


Fig. 2. Dry weight of *Ganoderma lucidum mycelia* in different culture systems with varying amounts of herbal medicine and time cultured.

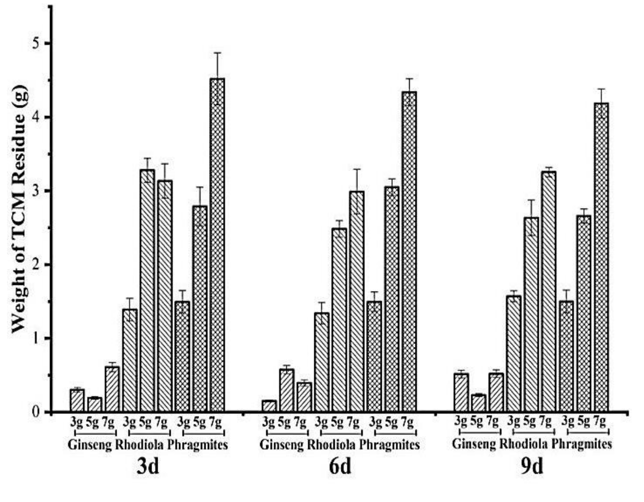


Fig. 3. Changes in the residues of the three herbal culture systems over varying dosage and time.

Figure 4(a) shows the variation of glucose content in the culture medium over time after inoculation of *Ganoderma* mycelium with ginseng, rhodiola, and phragmites. The addition of different types of herbs causes different levels of utilization of glucose by *Ganoderma lucidum*, as shown by the difference in colour after chromogenic reaction (Fig. 4a). The absorbance values were calculated to show the influence of the glucose content of the medium by the variation of herbal dosage and time in the form of bubble plots (Fig. 4b). The total sugar, glucose, and xylose contents and the cellulose, hemicellulose, and lignin contents expressed in bubble plots (Fig. 5).

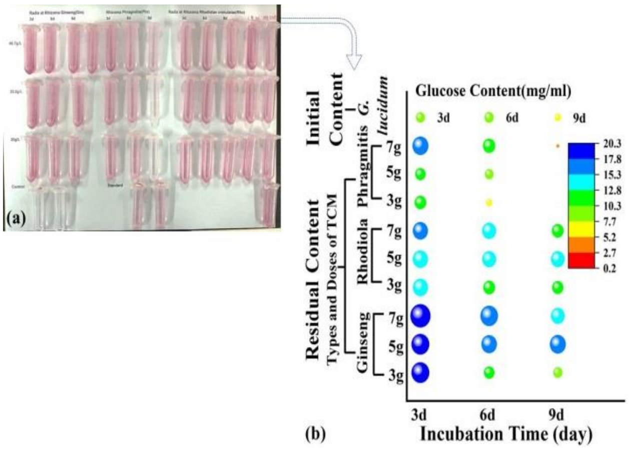


Fig. 4. Glucose variations in the three herbal culture systems over varying dosage and time.
(a) chromogenic reaction of glucose at different herbal dose - incubation times; (b) conversion of glucose content into bubble plots.

Different herbs have different "preferences" for the use of carbohydrates in the culture medium. The total sugar content of the systems containing ginseng and rhodiola increased as more herbs were added (Fig. 5a). Conversely, the total sugar content decreased with the increase in the number of days of incubation. The transformation of total sugar absorbance values was consistent with the control modifications, as can be shown. The total sugar content of the phragmites system did not change significantly. Compared to the control, the glucose content increased with all herbal addition, with the total sugar increasing more in the ginseng and phragmites systems. In contrast, the addition of rhodiola system showed almost no change in total sugar.

The glucose content in the culture medium with ginseng and phragmites increased with the addition of herbs and decreased with the increase in the number of days of incubation as seen by the conversion of glucose absorbance values, which is consistent with the control changes (Fig. 5b). There was almost no change in glucose over time in the rhodiola system. Compared to the control, the addition of different herbal medicines increased the glucose content, with the addition of ginseng increasing the glucose content the most, followed by rhodiola and phragmites increasing glucose content the least.

The D-xylose absorbance value conversion showed that the D-xylose content decreased with increasing days of incubation with both ginseng and phragmites was almost no change in xylose content with the addition of rhodiola (Fig. 5c). Compared to the control, the different herb's D-xylose content added increased, with a relatively large increase in the D-xylose content of ginseng and rhodiola and a small increase in D-xylose in phragmites compared to the control.

Semi-cellulose, and lignin are lower in ginseng. It can be seen that mycelium in the ginseng system uses the most cellulose, hemicellulose, and lignin of all the Chinese herbs. The next highest plant is phragmites, and the lowest is rhodiola. It can be seen that mycelium in the ginseng system uses the most cellulose, hemicellulose, and lignin of all the Chinese herbs. The next highest plant is phragmites, and the lowest is rhodiola.

Analysis of the causes of differences in culture systems: To compare the states of three different herbal medicines under the liquid culture conditions of *Ganoderma lucidum*, we analyzed the residue of the *Ganoderma*-TCM systems after 9 days of liquid culture by scanning electron microscopy. The results are shown in Figure 6.

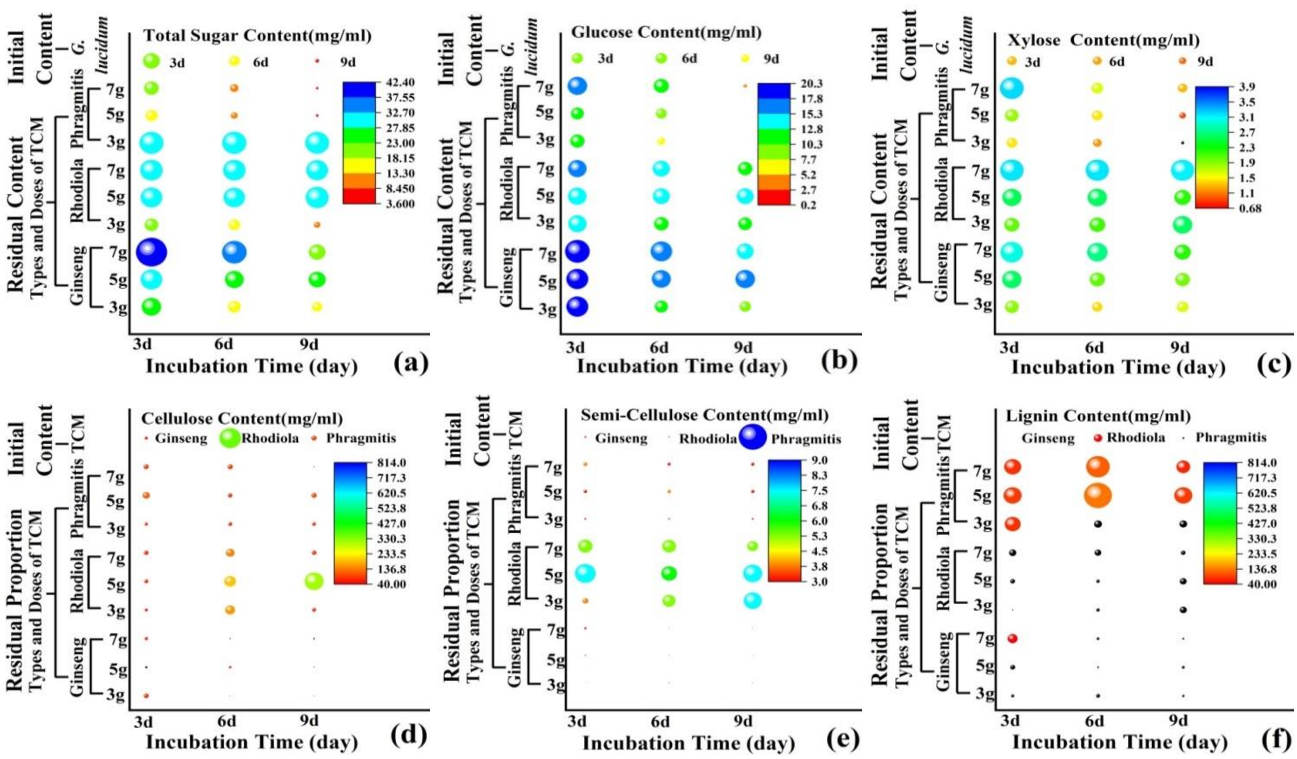
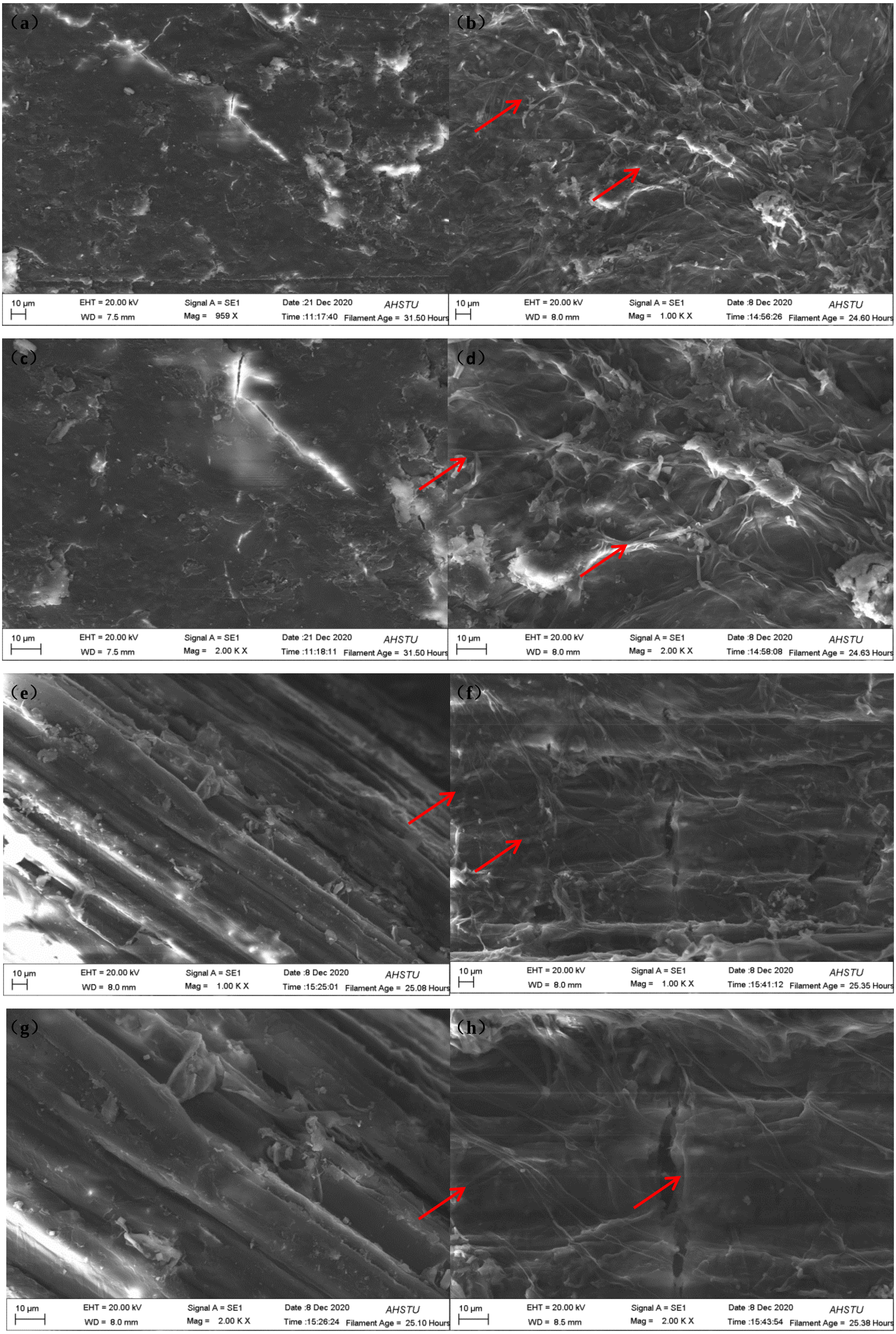


Fig. 5. Variation of sugars, lignin, and hemicellulose for the three herbal culture systems with varying dosage and time.
(a) Total sugars; (b) glucose; (c) xylose; (d) cellulose; (e) hemicellulose; (f) lignin.


(a)
10 µm
EHT = 20.00 kV
WD = 7.5 mm
Signal A = SE1
Mag = 959 X
Date :21 Dec 2020
Time :11:17:40
AHSTU
Filament Age = 31.50 Hours
(b)
10 µm
EHT = 20.00 kV
WD = 8.0 mm
Signal A = SE1
Mag = 1.00 K X
Date :8 Dec 2020
Time :14:56:26
AHSTU
Filament Age = 24.60 Hours
(c)
10 µm
EHT = 20.00 kV
WD = 7.5 mm
Signal A = SE1
Mag = 2.00 K X
Date :21 Dec 2020
Time :11:18:11
AHSTU
Filament Age = 31.50 Hours
(d)
10 µm
EHT = 20.00 kV
WD = 8.0 mm
Signal A = SE1
Mag = 2.00 K X
Date :8 Dec 2020
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AHSTU
Filament Age = 24.63 Hours
(e)
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AHSTU
Filament Age = 25.08 Hours
(f)
10 µm
EHT = 20.00 kV
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Date :8 Dec 2020
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AHSTU
Filament Age = 25.35 Hours
(g)
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EHT = 20.00 kV
WD = 8.0 mm
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Mag = 2.00 K X
Date :8 Dec 2020
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AHSTU
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(h)
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EHT = 20.00 kV
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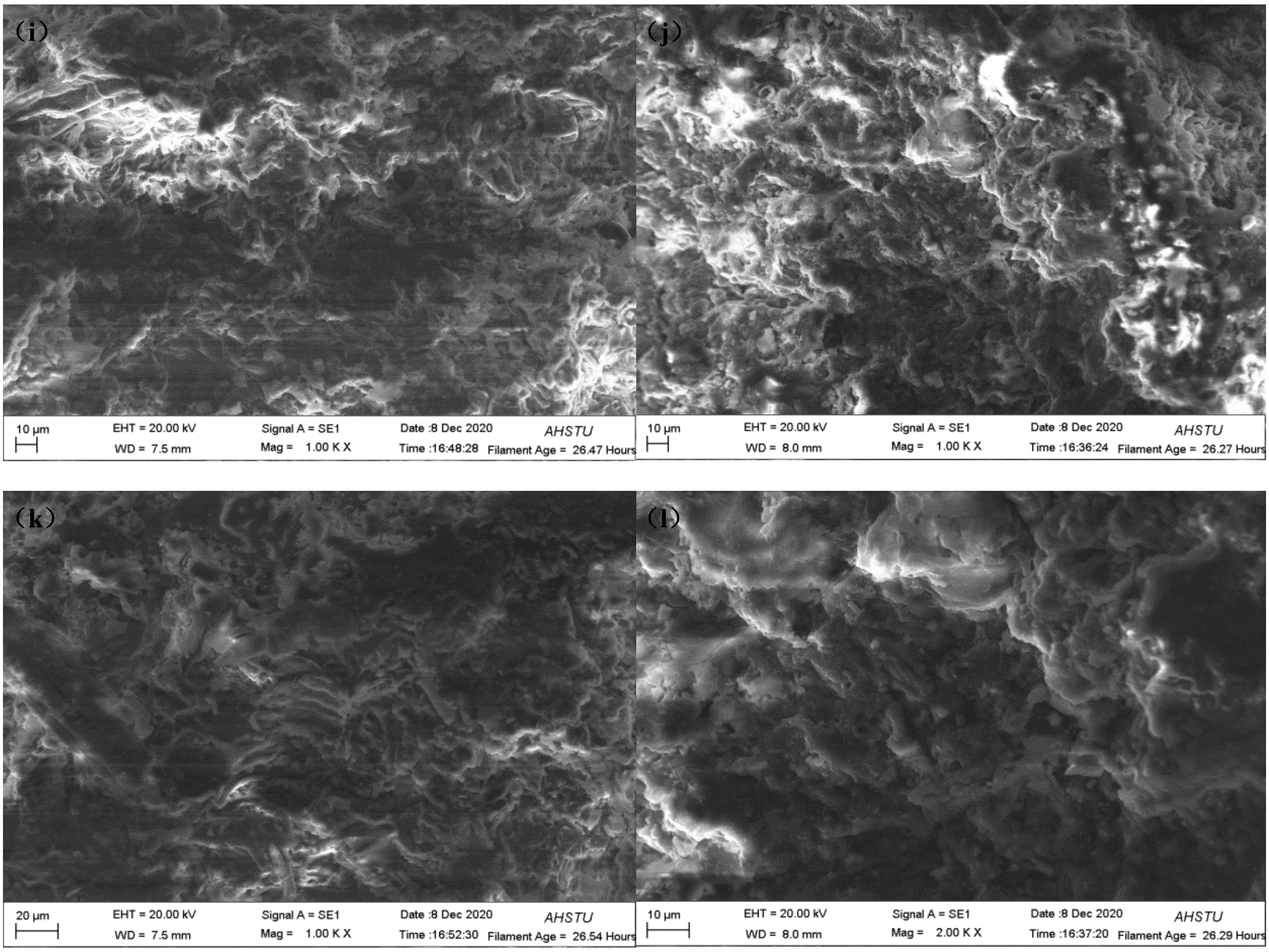

Fig. 6. Comparative electron microscopy photographs of different Chinese medicinal residues after 9 days of inoculation with *Ganoderma lucidum*.
(a) ginseng residue control (1000×); (b) ginseng-*Ganoderma* culture (1000×); (c) ginseng residue control (2000×); (d) ginseng-*Ganoderma* culture (2000×); (e) phragmites residue control (1000×); (f) phragmites-*Ganoderma* culture (1000×); (g) phragmites residue control (2000×); (h) phragmites-*Ganoderma* culture (2000×); (i) rhodiola residue control (1000×); (j) rhodiola-*Ganoderma* culture (1000×); (k) rhodiola residue control (2000×); (l) rhodiola-*Ganoderma* culture (2000×) *Ganoderma* mycelium at red arrows, so the *Ganoderma* mycelium can attach and use the ginseng residue as raw material to grow and develop.

After 9 days of liquid culture, *Ganoderma* mycelium was also attached to the surface of the phragmites (Figs. 6e-6h). Still, it was sparser than when compared to the ginseng residue. As seen in Figures 6(i)-(ii) (l), there was little change in rhodiola liquid culture and no *Ganoderma* mycelium growth on the surface, Figures 2 and 3 also show that this finding is correct. In rhodiola medium, no Ganoderma mycelium could be grown, and the use of rhodiola residue was minimal. This could be because the Ganoderma mycelium cannot rely on rhodiola for growth, or because some rhodiola components impede the Ganoderma mycelium's growth.

Discussion

Previous research has demonstrated that co-cultivating microorganisms (including Ganoderma lucidum) with TCM helps to produce and change some therapeutic components in TCM, and performs a role that TCM alone can't. Liquid culture of ginseng can be transformed to obtain ginsenoside compound K and 3-oxygen compound K, both inhibiting the growth of lung cancer cell line A549 in a dose-dependent manner (Jang *et al*., 2015); Ginsenoside Rg3 levels increased by 34.43 percent, and the fractions exert anti-cell prions (Liu *et al*., 2012); Ginsenoside Rg3 levels increased by 34.43 percent, and Luroot is used to alleviate fever and create humoral effects in traditional Chinese and Korean medicine (Byung-Soo 2014). Liu *et al*., (2008) demonstrated that reed rhizome can be employed in Ganoderma lucidum liquid culture, with a slight rise in mycelium biomass but less facilitation than cultured Semen Sojae Preparatum and Herba Lophatheri. (Panossian *et al*., 2010, Zhang *et al*., 2016) Rhodiola has pharmacological activity that includes boosting immunity, anti-fatigue, and antioxidant properties. Lactobacillus pentosus (Cui *et al*., 2016), Lactobacillus acidophillus (Sung *et al*., 2013), Candida utilis, Lactobacillus casei (Dai *et al*., 2017), and certain Endophytic Fungus (Cui *et al*., 2016) have all been employed in TCM fermentation investigations. Yoon cultured rhodiola-red ginseng with Lactobacillus acidophilus, indicating that a mixture of cultured hot water extracts protects muscle cells from cellular damage and oxidative stress generated by H_2O_2 (Yoon *et al*., 2013). However, research on the co-culture of rhodiola and Ganoderma lucidum is uncommon.

I. Rhizoma phragmitis had the highest concentration of semi-cellulose, rhodiola had the highest content of cellulose and lignin, and ginseng had the lowest content of cellulose, semi-cellulose, and lignin of the three traditional Chinese herbs. The effect on the Ganoderma lucidum mycelium was different when the three traditional Chinese medications were added to the culture system.Ginseng with *Ganoderma lucidum* strain (CGMCC 5.0026) has the best culture effect.

(i) After incubation, the mycelial biomass was the highest, with changes in an inverted U-shaped curve following increases in concentration; (ii) the amount of starch consumed was the highest; (iii) the least amount of ginseng residue remained after incubation; (iv) clear mycelial growth of Ganoderma lucidum could be seen in ginseng residue, as observed by scanning electron microscopy; (v) the contents of cerumen were the highest;

II. Phragmites aids Ganoderma lucidum in consuming cellulose, hemicellulose, and xylose.
The scanning electron microscope images show the growth of Ganoderma lucidum mycelium connected to the remaining residue. Ganoderma with phragmites rhizome (roots) has a little inferior culture than ginseng.

III. Ganoderma lucidum-rhodiola system's poor liquid culture effects
After incubation, only a few mycelia were visible to the naked eye, and electron micrographs revealed little evidence of mycelium occurrence or attachment. Rhodiola had the highest amount of Chinese medicine residue as well as the least amount of overall sugar consumption.
The culture outcomes in a liquid culture technique are affected by the characteristics of both the fungus strain and the herbal medicine, and they are not identical. We compared and analysed Ganoderma lucidum's interactions with three natural medications. The culture impact of Ganoderma lucidum strain CGMCC 5.0026 utilising ginseng was the best, according to the experimental results: I After incubation, the mycelial biomass was highest and showed an inverted U-shaped curve with increasing concentration; this result is consistent with Liu *et al*., (2012); (ii) the most starch was consumed; (iii) the least ginseng powder residue was observed after incubation; (iv) scanning electron microscopy revealed a clear picture of Ganoderm mycelia growth (2012).
Phragmites utilised xylose in the growth medium at a higher rate than ginseng and rhodiola. Scanning electron microscopy images revealed the growth of Ganoderma mycelium attached to the residual drug residue. Ginseng and rhodiola both promoted mycelium growth in phragmites.
The Ganoderma rhodiola culture was poor, with few mycelia visible to the naked eye and no evidence of mycelium or attachment in electron micrographs. Rhodiola has a weaker induction effect on Ganoderma than ginseng and phragmites, despite its high cellulose and lignin content. Further research is needed to determine the cause of this and the weakness of mycelial growth.

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